Modeling Cardiac Ischemia:
Hypoxia Induction for Cardioprotection Screening

Introduction

Myocardial ischemia, a pathological condition characterized by a reduced blood flow and subsequent decreased oxygen supply (hypoxia), can lead to cellular apoptosis/necrosis, arrhythmia, organ injury, and even death. While conventional therapies minimize cellular damage caused by hypoxia, morbidity and mortality remain high. Thus, relevant tools to develop effective therapeutic approaches for cardioprotection against and treatment of hypoxia are in demand.

iCell® Cardiomyocytes recapitulate the biochemical, electrophysiological, mechanical, and pathophysiological properties of native human cardiac myocytes. Derived from induced pluripotent stem (iPS) cells, these cardiomyocytes have demonstrated utility across a variety of life science applications (www.cellulardynamics.com/products/lit/). CDI has expanded this utility by developing a procedure for inducing hypoxia and quantifying related pathological outcomes. In addition to providing a model for ischemia-related research and drug development, iCell Cardiomyocytes can also be used to model and find potential therapeutic targets/pathways in the common co-morbidities of cardiac hypertrophy, diabetic cardiomyopathy, and dilated cardiomyopathy (1 - 3).

This Application Protocol describes how to induce and quantify hypoxia in iCell Cardiomyocytes as a human-based in vitro model of cardiac ischemia.

Required Equipment and Consumables

The following equipment and consumables are required in addition to the materials specified in the iCell Cardiomyocytes User's Guide.

Item	Vendor	Catalog Number
Equipment		
Hypoxia Chamber*	Multiple Vendors	
N_2 Tank with Regulator	Multiple Vendors	
Consumables		
iCell Cardiomyocytes	Cellular Dynamics International (CDI)	CMC-100-010-001 CMC-100-010-005
96-well Cell Culture Plates	Corning	3603
Creatine	Sigma	C0780
D-(+) Glucose Solution	Sigma	G8769
DMEM, No Glucose, No Glutamine, No Phenol Red	Life Technologies	A14430-01
GlutaMax	Thermo Fisher	35050-061
HEPES, 1 M Solution	Thermo Fisher	15630-106
L-carnitine	Sigma	C0283

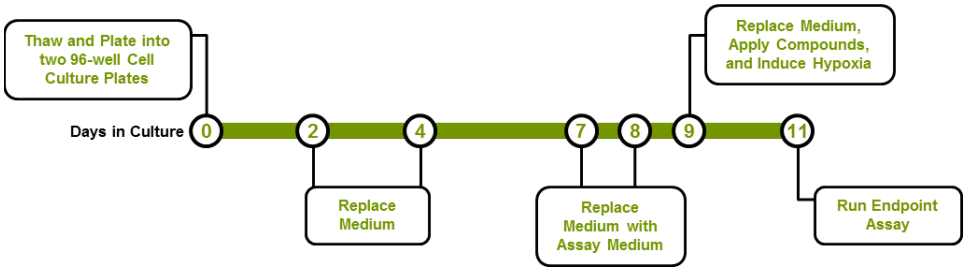
Item	Vendor	Catalog Number
Linoleic-oleic Acid	Sigma	L9655
Non-essential Amino Acids	Life Technologies	11140-050
Sodium Pyruvate	Thermo Fisher	11360-070
Taurine	Sigma	T8691

* Several hypoxia chambers are available. The data described here were obtained using the hypoxia chamber from BioSpherix, Cat. No. ProOx110.

Notes

Workflow

iCell Cardiomyocytes are thawed and plated into two gelatin-coated 96-well cell culture plates: one for hypoxia induction and one for normoxia control condition. On days 2 and 4 post-plating, spent medium is replaced with fresh Maintenance Medium. On day 7 post-plating, cardiomyocytes are washed, and spent medium is replaced with Assay Medium. On day 8 post-plating, spent medium is replaced with Assay Medium. On day 9 post-plating, spent medium is replaced with fresh Assay Medium, cardiomyocytes can be treated with compounds, and hypoxia is induced for 48 hours before running the endpoint assay. Weekend work can be avoided when cells are plated on a Monday.



Methods

Thawing iCell Cardiomyocytes

1. Coat two 96-well cell culture plates with 100 µl/well of 0.1% gelatin solution at 37°C for at least 1 hour according to the iCell Cardiomyocytes User's Guide.

   Note: *This Application Protocol provides instructions for preparing two 96-well cell culture plates: one for hypoxia induction and one for normoxia control condition.*

   Note: *1 unit of iCell Cardiomyocytes contains enough cells to plate into one 96-well cell culture plate. Thaw the number of units of iCell Cardiomyocytes necessary for your experimental plans.*

2. Thaw iCell Cardiomyocytes according to the User's Guide.
3. Remove a sample of cells to perform a cell count using a hemocytometer (using trypan blue exclusion to identify viable cells) or an automated cell counter.
4. Dilute the cell suspension in iCell Cardiomyocytes Plating Medium (Plating Medium) to 200,000 plated cells/ml. Refer to the User's Guide for instructions to calculate the *Target Plating density* based on *Plating Efficiency.*

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5. Aspirate the gelatin solution. Immediately add 100 µl/well of the cell suspension (20,000 cells/well) into the two 96-well cell culture plates.
6. Culture iCell Cardiomyocytes in a cell culture incubator at 37°C, 5% CO_2.

Maintaining iCell Cardiomyocytes

1. Maintain iCell Cardiomyocytes according to the User's Guide, replacing spent medium with iCell Cardiomyocytes Maintenance Medium (Maintenance Medium) on days 2 and 4 post-plating.
2. Culture iCell Cardiomyocytes in a cell culture incubator at 37°C, 5% CO_2.

Inducing Hypoxia in iCell Cardiomyocytes

iCell Cardiomyocytes require a medium replacement on days 7 and 8 post-plating to equilibrate the cells to the Assay Medium. The cardiomyocytes are then suitable for inducing hypoxia on day 9 post-plating. The highest response to hypoxia is achieved when the cells are maintained in hypoxic conditions for 48 hours. This timing allows for response detection on day 11 post-plating.

1. Prepare and store the Assay Medium.
   a. Combine the following components except for the linoleic-oleic acid:

Component	Amount	Final Concentration
DMEM, No Glucose, No Glutamine, No Phenol Red	465 ml	93%
Creatine	328 mg	5 mM
D-(+) Glucose Solution, 2.5 M, 450 g/l	550 µl	2.75 mM
GlutaMax, 100X	5 ml	1X
HEPES, 1 M	5 ml	10 mM
L-carnitine, 200 mM	5 ml	2 mM
Non-essential Amino Acids, 100X	5 ml	1X
Sodium Pyruvate, 100X	5 ml	1X
Taurine, 500 mM	5 ml	5 mM
Linoleic-oleic Acid, 100X	5 ml	1X

   b. Filter the medium using a 0.22 µm filter.
   c. Add the linoleic-oleic acid to complete the medium.
   d. Prepare 20 ml working aliquots of the medium.
   e. Store the medium at 4°C for up to 2 weeks.
2. Equilibrate 2 aliquots of Assay Medium at room temperature on day 7 post-plating.
3. Remove the 96-well cell culture plates containing iCell Cardiomyocytes from the cell culture incubator and wash cells twice with 100 µl/well of Assay Medium to remove the spent Maintenance Medium.
4. Equilibrate an aliquot of Assay Medium at room temperature and replace the spent medium with 100 µl/well of Assay Medium on day 8 post-plating.
5. Prepare the hypoxia chamber and equilibrate to 1% O_2 in a cell culture incubator at 37°C, 5% CO_2 on day 9 post-plating.

6. Equilibrate an aliquot of Assay Medium at room temperature and replace the spent medium with 90 µl/well of Assay Medium for compound application.
7. Prepare test compound dilutions in Assay Medium at 10X the final concentration in a clean 96-well cell culture plate (compound plate).

   Note: *Final DMSO concentrations above 0.1% should be used with caution. Therefore, if test compounds are dissolved in DMSO, the 10X compound solutions should not exceed 1% DMSO.*
8. Transfer 10 µl/well of the 10X compound dilutions from the compound plate to 96-well cell culture plates containing iCell Cardiomyocytes.
9. Incubate one cell culture plate in the equilibrated hypoxia chamber for 48 hours. Incubate the other cell culture plate in a standard cell culture incubator at 37°C, 5% CO_2 for normoxia control condition.
10. Perform the endpoint assay according to manufacturer's instruction after 48 hours of incubation.

Example Data

Figures 1 and 2 illustrate representative assay data for evaluating hypoxia-induced pathology and its rescue.

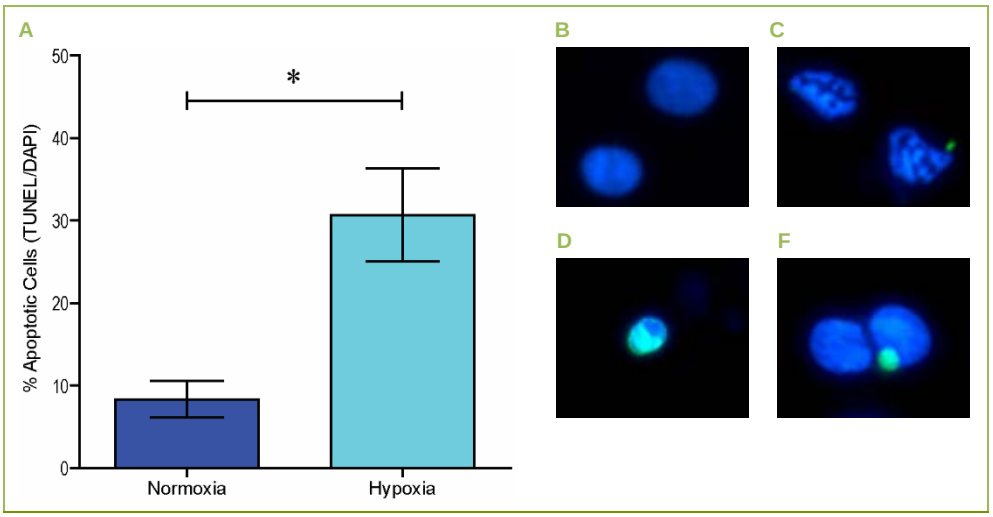

Figure 1: iCell Cardiomyocytes Provide a Human-based Model of Hypoxia
*Hypoxia-induced apoptosis is quantified by (A) the number of cells positive for TUNEL/DAPI (mean ± SEM, * $p < 0.05$). TUNEL (green) and DAPI (blue) staining illustrates (B) normal nuclear morphology under control conditions and (C) nuclear fragmentation and (D, E) chromatin condensation under hypoxia.*

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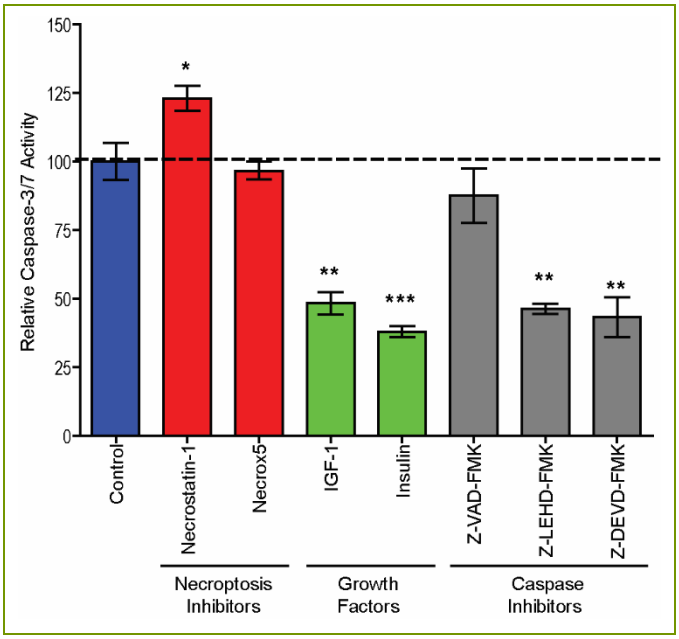

Figure 2: iCell Cardiomyocytes Can Be Used to Screen for Cardioprotective Compounds

*Inhibitors and growth factors were added immediately before induction of hypoxia, and the cellular response measured 48 hours later by Caspase-Glo 3/7 (Promega). iCell Cardiomyocytes were treated with growth factors, and a couple of the caspase inhibitors that were tested displayed significantly lower caspase activity suggesting a cardioprotective effect against hypoxia. Necrosis inhibitors had little or no effect (mean ± SEM. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$).*

Summary

iCell Cardiomyocytes, derived from human iPS cells, provide an in vitro cellular system for modeling cardiac ischemia by induction of hypoxia and quantification of cellular endpoints. The methods and data presented here highlight an HTS-compatible 96-well assay format for screening for cardioprotective molecules and/or conditions that could enable the discovery of novel potential therapeutic interventions.

References

1. Carlson C, Koonce C, et al. (2013) Phenotypic Screening with Human iPS Cell-derived Cardiomyocytes: HTS-compatible Assays for Interrogating Cardiac Hypertrophy. J Biomol Screening **18**(10):1203-11.
2. Drawnel FM, Boccardo S, et al. (2014) Disease Modeling and Phenotypic Drug Screening for Diabetic Cardiomyopathy Using Human Induced Pluripotent Stem Cells. Cell Rep **9**(3):810-21.
3. Traister A, Li M, et al. (2014) Integrin-linked Kinase Mediates Force Transduction in Cardiomyocytes by Modulating SERCA2a/PLN Function. Nature Comm **5**:4533.

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